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The effect of temperature on Triclosan and Lead exposed mussels☆

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ABSTRACT

Interest on the effects of emerging contaminants over aquatic organisms has increased in the last years. Nonetheless, the toxic action of classical natural and anthropogenically-driven metals has also to be monitored, especially because they reflect real environmental situations. For that, in the present study we focused on the effects on the marine mussel *Mytilus galloprovincialis* of the personal care product Triclosan (TCS) and Lead (Pb), as toxic metal, under separate and co-exposure situations at environmentally relevant concentrations: TCS (1 μg/L) and Pb (50 μg/L). The consideration of an additional factor such as an increase in ambient temperature was also included to provide a forecasted scenario of climate change: from the ambient temperature at actual conditions (17 °C) to a predicted warming situation (22 °C). Water chemical characterization and some physical properties and bioaccumulation of TCS and Pb in mussels at the end of the experiment (28 days) was considered. The parameters followed up comprise the energy related system production (electron transport system) and glycogen and protein reserves. Antioxidant enzymatic defences towards reactive oxygen species (ROS) and the consequences of ROS damage over endogenous lipids (LPO) and proteins (PC). Overall the results suggested only particular responses to chemical exposures at 17 °C whereas at 22 °C the detoxification machinery was set up and this prevented the occurrence of LPO. Nonetheless, PC formation occurred under Pb and TCS + Pb co-exposure at the highest temperature. Due to the complexity of the study: 4 chemical conditions, 2 temperatures and 10 biomarkers considered, a principal component ordination (PCO) analysis was included. The results of this integrative analysis confirmed a clear effect of the temperature, more responsiveness to drugs at 22 °C and in all likelihood due to Pb presence.

1. Introduction

A large variety of man-made substances reach the aquatic environment, including the ones of increasing environmental concern used as personal care products (PCPs). One of the most common PCPs detected in the environment worldwide is Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether, TCS), an antimicrobial agent used as an ingredient in household items, personal care products (e.g. cosmetics, toothpastes, mouthwashes, deodorants and shampoos) and medical devices (Dann and Hontela, 2011). In September 2016, TCS was banned from soap products following the risk assessment by the U.S.A. Food and Drug Administration (FDA). Also, the European Union (EU) banned the availability and use on the EU market of biocidal products containing TCS, starting January 2017. However, TCS still remains, at high concentrations, in other personal care products such as toothpaste, mouthwash, hand sanitizer, and surgical soaps (Weatherly and Gosse, 2017). Although wastewater treatment plants (WWTPs) are usually highly efficient removing TCS, as a consequence of its extensive use a significant portion of this antimicrobial or its metabolic degradation products is discharged into receiving waters, including lakes, rivers and finally reaching coastal and estuarine waters, where TCS may be found at concentrations ranging from hundreds of ng/L to few μg/L (Dhillon et al., 2015; Ebele et al., 2017). As a consequence, continuous exposure of aquatic organisms to TCS has led to detectable levels in a number of aquatic species, with potential harmful consequences, including in algae, invertebrates, fish and marine mammals (Bedoux et al., 2012; Dann and Hontela, 2011; Tamura et al., 2013).

In addition to these so-called emerging substances, aquatic

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environments are sink of classical pollutants, namely metals that have been of great environmental concern all over the world due to their toxicological impacts towards biological resources and humans. Although metals may reach the aquatic systems through natural geological processes, such as rock erosion, their presence can be exacerbated by anthropogenic activities, including discharges from WWTPs or industry (review by Tchounwou et al., 2012). Among existing metals, lead (Pb) is one of the most studied. Pb has been largely and extensively used in battery cases, paints and as an additive in gasoline that even at low concentrations can bioaccumulate and be toxic to aquatic organisms (Company et al., 2008; Krause-Nehring et al., 2012). Damages induced by Pb, including in bivalves, are usually achieved through inhibition of essential enzymes comprising those forming the cellular antioxidant pool. Therefore, lower removal of reactive oxygen species by antioxidant enzymes can indirectly lead to lipid peroxidation of cell membranes (Aouini et al., 2018; Meng et al., 2018).

Aquatic systems are also subjected to environmental changes related climate changes (Gazeau et al., 2013; Hoffmann et al., 2012) that are likely to impact aquatic organisms such as bivalves (Freitas et al., 2016; 2017; Lannig et al., 2010; Matoo et al., 2013; Moreira et al., 2018; Munari et al., 2018). In the scenario of global warming, aquatic organisms are subjected to water temperature fluctuations that may alter their thermal tolerances. This is particularly relevant in polluted environments, where increased temperatures could also modulate the susceptibility of organisms to pollutants (Acevedo-Whitehouse and Duffus, 2009). Among aquatic species, bivalves are considered to be mostly at risk due to the combined effects of temperature and habitat quality because they are sessile and many of them thrive in shallow coastal waters where changes in thermal gradients are more accused (Anestis et al., 2007; Dimitriadis et al., 2012; Gagne et al., 2006). It has already been demonstrated that temperature alone, can affect the metabolic rates and oxidative status of aquatic organisms (Coppola et al., 2017; Velez et al., 2017). In addition to direct temperature impacts in aquatic organisms may change their sensitivity towards pollutants, through alterations in the rate of biochemical and physiological processes, but may also change pollutants bioavailability and toxicity (Attig et al., 2014; Bat et al., 2000; Coppola et al., 2018; Ivanina et al., 2009; Khan et al., 2006, 2007; Lannig et al., 2006; Mubiana and Blust, 2007; Sokolova and Lannig, 2008). Still, little is known on the combined effects of climate change and emerging pollutants such as PCPs, and especially concerning mixtures including both classical toxic metals and PCPs. While an increasing body of knowledge has demonstrated the physiological and biochemical impacts induced by warming and pollutants in bivalves (see reference above), little information is known on the alterations caused in a model bivalve by both stressors acting in combination. For that, mussels *Mytilus galloprovincialis* were exposed for 28 days to environmentally realistic concentrations of two pollutants: TCS and Pb individually and in a mixture, under the actual temperature at the time of sampling (17 °C) and a predicted warming scenario (22 °C). Biomarkers related to metabolic capacity and oxidative stress were evaluated. The metabolic parameters imply energy expenditure costs and therefore are physiologically relevant and those measured at the molecular and/or cellular level constitute sensitive early warning tools for assessing biological effects caused by environmental stressors.

2. Material and methods

2.1. Experimental conditions

Mytilus galloprovincialis specimens were collected in the Ria de Aveiro (northwest Atlantic coast of Portugal), in April 2018. In order to minimize the effect of body weight, organisms with similar weight (25.5 ± 7.5 g) were selected. Mussels were maintained in the laboratory for 15 days before conducting the experiment in order to release metals and microorganisms (Freitas et al., 2012; Maffei et al., 2009). During this period, organisms were maintained at 17.0 ± 1.0 °C; pH 7.80 ± 0.10, 12 h light: 12 h dark photoperiod under continuous aeration, in artificial seawater (salinity 30 ± 1) prepared with commercial salt (Tropic Marin® SEA SALT from Tropic Marine Center) and deionized water. Seawater was renewed every two days. After this adaptation period, organisms were distributed into different glass aquaria (7 L seawater, salinity 30), with seven individuals per aquarium and three aquaria per treatment, in two different climatic rooms set at 17 °C and 22 °C.

At each temperature (17 and 22 °C) the tested treatments were: a) control (CTL), without Triclosan (TCS) and Lead (Pb); b) TCS 1.0 µg/L (TCS); c) Pb 50.0 µg/L (Pb) and d) TCS 1.0 µg/L and Pb 50 µg/L (TCS + Pb).

Concentrations of Pb and TCS were selected taking into consideration the presence of both chemicals in polluted areas (among others, Srichandan et al., 2016; Vázquez-Sauceda et al., 2012). Taking into consideration the average temperature of the sampling area during April (17–20 °C, IPMA, 2018) 17 °C was selected as control temperature. Considering annual range of average temperatures (13.4–22.9 °C) for *M. galloprovincialis* habitats in the Ria de Aveiro (Coelho et al., 2014; Velez et al., 2015) and the fact that a 2–4 °C rise in water temperature is predicted in the current century (IPCC, 2014), the temperature of 22 °C was considered to realistically represent warming conditions.

The experiment lasted 28 days, and mussels were collected at the end of the exposure. During this period, aquaria were continuously aerated; temperature and salinity were checked daily with a thermometer and a refractometer, respectively and when necessary readjusted. Mortality was checked daily and organisms were considered dead when their shells gaped and failed to shut again after external stimulus. During the entire exposure period animals were fed with Algamac protein plus (150.000 cells/animal) twice a week and exposure medium (seawater at salinity 30) was renewed weekly, after which TCS and Pb concentrations were re-established. At the end of the exposure, mussels were frozen individually and manually homogenized with a mortar and a pestle in liquid nitrogen. Each homogenized organism was divided into 0.5 g fresh weight (FW) aliquots, before being used for biomarker and chemical analyses.

2.2. Triclosan and lead quantification

Triclosan concentrations in water were measured following Cheng et al. (2011) protocol. The extraction was performed with C-18 SPE cartridges (HYPERSEP, 6 mL, 1 g, Thermo Scientific). Each cartridge was pre-conditioned with 6 mL of dichloromethane, and 6 mL of methanol and then rinsed with 6 mL of ultrapure water by the use a SPE manifold (Thermo Scientific). Samples were acidified to pH 3 (Cheng et al., 2011), and passed through the cartridges with the aid of vacuum. Then, the cartridges were washed with 4 mL 20% methanolic solution and air-dried under vacuum for 20 min. TCS was eluted from the cartridges with 8 mL of dichloromethane and the extracts were completed dried with a rotavapor. The residue was redissolved in 0.2 mL of toluene and analysed by GC–MS.

Triclosan was extracted from soft tissue as suggested by Schmidt and Snow (2016) using a QuEChERS method. Ultrapure water was added to an aliquot of sample tissue into a 50 mL polypropylene tube and the mixture was manually shaken. Acetonitrile was successively poured and the tube was still shaken by hand. Then, QuEChERS extraction salts kit (Agilent Technologies) was added and the extraction mixture was vigorously shaken again. The centrifuged extract was finally treated with QuEChERS purification salts kit, filtered and concentrated in a 2 mL vial for GLC analysis. The extracts were then processed according to the GC–MS method reported by Tohidi and Cai (2015). Standard stock solutions of TCS (Sigma-Aldrich) were prepared in dichloromethane (concentration range: 0.1–1.2 mg/L) and stored at < 6 °C. Triclosan analyses were performed with a GC Trace 1300 (Thermo Scientific) coupled to a TriPlus RSH autosample and a triple

quadrupole mass spectrometer TSQ Duo with an electron impact ionization source (EI) (Thermo Scientific). The GC was equipped with an Agilent DB-5MS column. The analysis was performed in SIM technique. Highly pure helium was used as carrier gas for the GC analyses. The detection limit (LOD), calculated as a signal-to-noise ratio of 3:1, was 0.008 μg/L for water samples and 0.13 ng/g dry weight (d.w) for soft tissues samples. The recovery was > 91% for water samples and > 89% for soft tissues.

Concentrations of Pb in water and tissue samples were determined with an AAS Varian SpectrAA240Z following the EPA 7010 method (2007). Lead standard was purchased from Titolchimica (Italy). Water samples were directly analysed prior acidification with nitric acid at pH 2. Soft tissues were dried at 40 °C for 48 h and digested following the method EPA 3051A (2007) by the use of an Ethos 1 Microwave Digestion System (Milestone, FKV). The LOD of water samples was 0.8 μg/L and 0.4 μg/g d.w. for soft tissues samples. The percent of recovery was > 98% for waters and > 81% for tissues.

2.3. Biomarkers

Biomarkers were determined in organisms whole soft tissue. For each biochemical determination, 0.5 g FW soft tissue per organism was used (3 individuals per replicate and 9 per treatment). For each treatment, the markers selected are related to energy production and usage: electron transport system (ETS) activity; glycogen (GLY) and total protein (PROT) content, oxidative stress: superoxide dismutase (SOD); catalase (CAT); glutathione peroxidase (GPx); glutathione reductase (GRed); and glutathione S-transferases (GSTs) activities, and cellular damage: lipid peroxidation (LPO) and protein carboxylation (PC) levels. All biochemical parameters were performed in duplicate using a microplate reader (Biotek). The extraction for each biomarker was performed with specific buffers. For ETS activity assay supernatants were extracted in 0.1 M Tris-HCl buffer. For LPO assay supernatants were extracted in 20% trichloroacetic acid (TCA). For GRed assay samples were extracted in 0.1 M dipotassium phosphate. For SOD, CAT, GSTs, GPx, PC, PROT and GLY assays supernatants were extracted in phosphate buffer. Samples were homogenized for 15 s at 4 °C and centrifuged for 10 min at 10,000*g* (or 3000*g* for ETS) at 4 °C. Supernatants were stored at −80 °C or immediately used for analysis.

2.3.1. Energy-related markers

The ETS activity was measured based on King and Packard (1975) and the modifications performed by De Coen and Janssen (1997). Absorbance was measured during 10 min at 490 nm with intervals of 25 s and the extinction coefficient of 15,900 $M^{-1}\,cm^{-1}$ was used to calculate the amount of formazan formed. Results were expressed in nmol per min per g of fresh weight (FW).

For GLY quantification the sulphuric acid method was used, as described by Dubois et al. (1956). Glucose standards were used (0–10 mg/mL) to produce a calibration curve. Absorbance was measured at 492 nm after incubation during 30 min at room temperature. Results were expressed in mg per g FW.

The PROT content was determined according to the spectrophotometric Biuret method (Robinson and Hodgen, 1940). Bovine serum albumin (BSA) was used as standard calibration curve (0–40 mg/mL). Absorbance was read at 540 nm. The results were expressed in mg per g FW.

2.3.2. Oxidative stress markers

SOD activity was determined by the Beauchamp and Fridovich (1971) method after adaptations performed by Carregosa et al. (2014). The standard curve was formed using SOD standards (0.25–60 U/mL). Samples' absorbance was read at 560 nm after 20 min of incubation at room temperature. Results were expressed in U per g FW where one unit (U) represents the quantity of the enzyme that catalyzes the conversion of 1 μmol of substrate per min.

CAT activity was quantified according to the Johansson and Borg (1988) method and the modifications performed by Carregosa et al. (2014). The standard curve was determined using formaldehyde standards (0–150 μM). Absorbance was measured at 540 nm. The enzymatic activity was expressed in U per g of FW, where U represents the amount of enzyme that caused the formation of 1.0 nmol formaldehyde per min at 25 °C.

GPx activity was quantified following Paglia and Valentine (1967). The absorbance was measured at 340 nm in 10 s intervals during 5 min and the enzymatic activity was determined using $\varepsilon = 6.22\,mM^{-1}\,cm^{-1}$. The results were expressed as U per g FW, where U represents the number of enzymes that caused the formation of 1.0 μmol NADPH oxidized per min.

GRed activity was determined using the method described in Carlberg and Mannervik (1985). The absorbance was measured at 340 nm and the enzymatic activity was determined using $\varepsilon = 6.22\,mM^{-1}\,cm^{-1}$. The results were expressed as U per g FW, where U represent the enzymes amount that caused the formation of 1.0 μmol NADPH oxidized per min.

GSTs activity was quantified following Habig et al. (1974) protocol with some adaptations performed by Carregosa et al. (2014). GSTs activity was measured spectrophotometrically at 340 nm ($\varepsilon = 9.6\,mM^{-1}\,cm^{-1}$). The enzymatic activity was expressed in U per g of FW where U is defined as the amount of enzyme that catalysis the formation of 1 μmol of dinitrophenyl thioether per min.

2.3.3. Cellular damage markers

LPO determination was done following the method described by Ohkawa et al. (1979). LPO levels were measured trough the quantification of malondialdehyde (MDA), a by-product of lipid peroxidation. Absorbance was measured at 535 nm and the extinction coefficient of 156 $mM^{-1}\,cm^{-1}$ was used to calculate LPO levels, expressed in nmol of MDA formed per g of FW.

The quantification of carbonyl groups in oxidized proteins (PC) was done following the 2,4-dinitrophenylhydrazina (DNPH) alkaline method, described by Mesquita et al. (2014). Absorbance was measured at 450 nm and the extinction coefficient of 22,308 $M^{-1}\,cm^{-1}$ was used to calculated PC levels, expressed in nmol per g of FW.

2.4. Statistical analyses

Biochemical parameters obtained from each treatment were submitted to statistical hypothesis testing using permutational analysis of variance, employing the PERMANOVA+ add-on in PRIMER v6 (Anderson et al., 2008). In figures, for each biochemical parameter, significant differences ($p \leq 0.05$) among treatments (CTL 17 °C, TCS 17 °C, Pb 17 °C, TCS + Pb 17 °C, CTL 22 °C, TCS 22 °C, Pb 22 °C, TCS + Pb 22 °C) were identified with lowercase letters for temperature 17 °C and upper case letters for temperature 22 °C. In figures, for each treatment significant differences between exposure temperatures (17 °C and 22 °C) are presented with asterisks.

The matrix gathering the biochemical descriptors per treatment were used to calculate the Euclidean distance similarity matrix. This similarity matrix was simplified through the calculation of the distance among centroids matrix based on the treatment, which was then submitted to ordination analysis, performed by Principal Coordinates (PCO). Pearson correlation vectors of biochemical descriptors (correlation > 0.75) were provided as supplementary variables being superimposed on the top of the PCO graph.

Table 1
Triclosan (TCS) and Lead (Pb) concentrations (μg/L) in water samples collected immediately after spiking every week along the experimental period (28 days) and in each tested condition. Triclosan LOD 0.008 μg/L. Lead LOD 0.8 μg/L.

		1st week		2nd week		3rd week		4th week	
		TCS	Pb	TCS	Pb	TCS	Pb	TCS	Pb
17 °C	CTL	< LOQ	9.74	< LOQ	11.32	< LOQ	10.46	< LOQ	9.51
	Pb	< LOQ	55.86	< LOQ	54.81	< LOQ	58.12	< LOQ	59.96
	TCS	0.93	11.12	1.13	11.26	1.06	9.94	0.96	9.77
	TCS + Pb	0.81	57.11	0.78	56.15	0.96	51.16	0.91	55.18
22 °C	CTL	< LOQ	8.14	< LOQ	7.72	< LOQ	7.87	< LOQ	7.91
	Pb	< LOQ	53.2	< LOQ	52.18	< LOQ	56.97	< LOQ	58.87
	TCS	0.84	10.23	0.97	9.44	0.83	11.21	0.83	9.62
	TCS + Pb	0.79	55.23	0.88	54.79	0.85	48.67	0.94	51.34

Table 2
Triclosan (TCS) and Lead (Pb) concentrations in mussels collected at the end of the experimental period (28 days) and in each tested condition. Triclosan LOD 0.13 ng/g. Lead LOD 0.40 μg/g. Different letters (a, b and c) represent significant differences ($p \leq 0.05$) among conditions.

		TCS (ng/g)	Pb (μg/g)
17 °C	CTL	< LOQ	1.40 ± 0.28^{a}
	TCS	13.88 ± 1.46^{a}	1.20 ± 0.04^{a}
	Pb	< LOQ	2.32 ± 0.11^{b}
	TCS + Pb	15.96 ± 2.97^{a}	2.19 ± 0.12^{b}
22 °C	CTL	< LOQ	1.40 ± 0.28^{a}
	TCS	10.22 ± 1.78^{a}	1.38 ± 0.42^{a}
	Pb	< LOQ	2.32 ± 0.12^{b}
	TCS + Pb	12.99 ± 3.07^{a}	2.97 ± 0.14^{c}

3. Results

3.1. Triclosan and lead levels in water and mussels soft tissue

Concentrations of TCS and Pb were weekly measured in the exposure medium soon after spiking and were similar to the desired nominal concentrations of 1 μg/L and 50 μg/L, respectively (Table 1). These results validated the spiking process.

Concentrations of TCS and Pb in mussels soft tissues at the end of the exposure period (28 days) are presented in Table 2. The results showed that in control mussels presence of TCS was lower than the quantification limit (0.40 ng/g d.w), while in TCS exposed mussels alone or combined with Pb, the concentrations were higher (8.44–18.93 ng/g d.w) regardless of temperature. In what regards to Pb, the concentrations found (1.1–3.1 μg/g d.w) were significantly higher in exposed mussels (alone or combined with TCS) in comparison to non-contaminated mussels and values were even higher under the combined exposure at higher temperature (22 °C).

3.2. Biochemical markers

3.2.1. Energy-related parameters

ETS activity was significantly decreased in mussels exposed to TCS, Pb and the combination of both, at the two temperatures tested (17 and 22 °C). ETS values observed at 22 °C were significantly higher than those at 17 °C, except for mussels exposed to TCS (Fig. 1A).

Reserves of GLY content showed no significant differences among treatments and temperatures (Fig. 1B). PROT content showed no significant differences among treatments in mussels maintained at 17 °C, while it decreased significantly in contaminated mussels (TCS, Pb and TCS + Pb) at 22 °C. Significantly higher values for PROT reserves were observed in mussels exposed to 22 °C compared to 17 °C, except for those exposed to Pb (Fig. 1C).

3.2.2. Oxidative stress markers

SOD activity showed a significant increase in mussels exposed to Pb and TCS + Pb only at 22 °C with respect to control and TCS alone exposed organisms. SOD activity was significantly lower in organisms exposed to Pb and TCS + Pb at 17 °C in contrast to the same treatments at 22 °C (Fig. 2A).

CAT activity showed no significant differences among treatments in mussels at 17 °C, while at 22 °C contaminated mussels (TCS, Pb and TCS + Pb) showed significantly lower CAT activity in comparison to controls (Fig. 2B).

GPx activity in mussels maintained at 17 °C was significantly lower when exposed to TCS alone. By contrast, at 22 °C, an opposite pattern was observed with significantly higher activity in organisms exposed to TCS in comparison to the remaining treatments. Comparing temperature effects at the four treatments, higher activity was observed in mussels exposed to TCS and 22 °C, while at the remaining conditions GPx activity was significantly higher at 17 °C (Fig. 2C).

GRed activity in mussels kept at 17 °C significantly increased in those exposed to Pb while at 22 °C significantly higher activities were observed in all contaminated organisms in comparison to the controls. Comparing both temperatures, significant higher activity was observed at 17 °C in control organisms and in those exposed to Pb (Fig. 2D).

Regarding to GSTs activity, mussels exposed to 17 °C showed no significant differences among treatments, while at 22 °C contaminated mussels significantly decreased the activity of this family of enzymes. When comparing both temperatures, significantly higher activity was achieved at 22 °C in all treatments (Fig. 3).

3.2.3. Cellular damage markers

Mussels maintained at 17 °C showed significantly higher LPO values when exposed to TCS, Pb and their combination (TCS + Pb) while at temperature 22 °C no significant differences were observed among treatments. No significant differences were observed between the two temperatures in any treatment (Fig. 4A).

Regarding PC levels, mussels maintained at 17 °C showed no significant differences among treatments, while those kept at 22 °C showed significantly higher levels when exposed to Pb and TCS + Pb. Comparing both temperatures, significantly higher PC levels were obtained at 22 °C in mussels exposed to Pb and TCS + Pb (Fig. 4B).

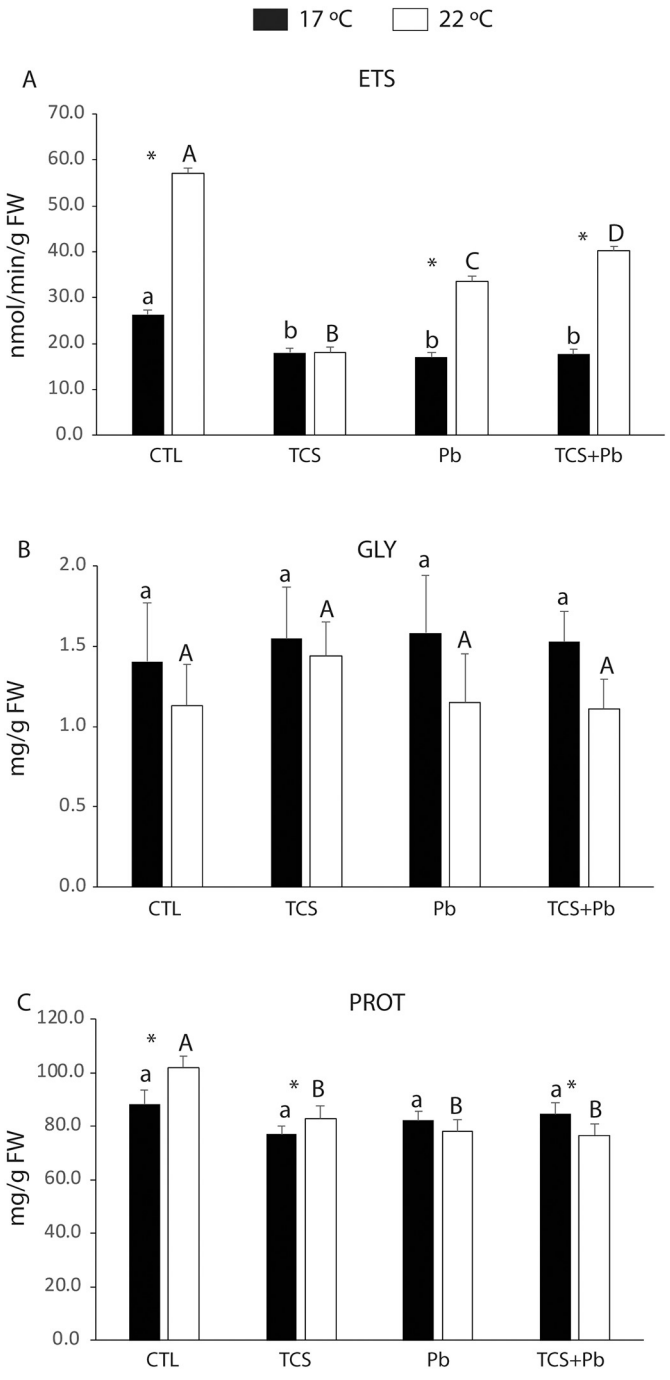

Fig. 1. A) Electron transport system (ETS) activity; B) Glycogen (GLY) and C) Protein (PROT) concentrations, in *Mytilus galloprovincialis* after 28 days-exposure. At each temperature (17 and 22 °C) the tested treatments were: control (CTL), without Triclosan (TCS) and Lead (Pb); TCS 1.0 µg/L (TCS); Pb 50.0 µg/L (Pb); TCS 10.0 µg/L and Pb 50 µg/L (TCS + Pb). Significant differences among treatments are represented with different letters: lower case letters for 17 °C and upper case letters for 22 °C. Differences between both temperatures for each treatment are represented with an asterisk.

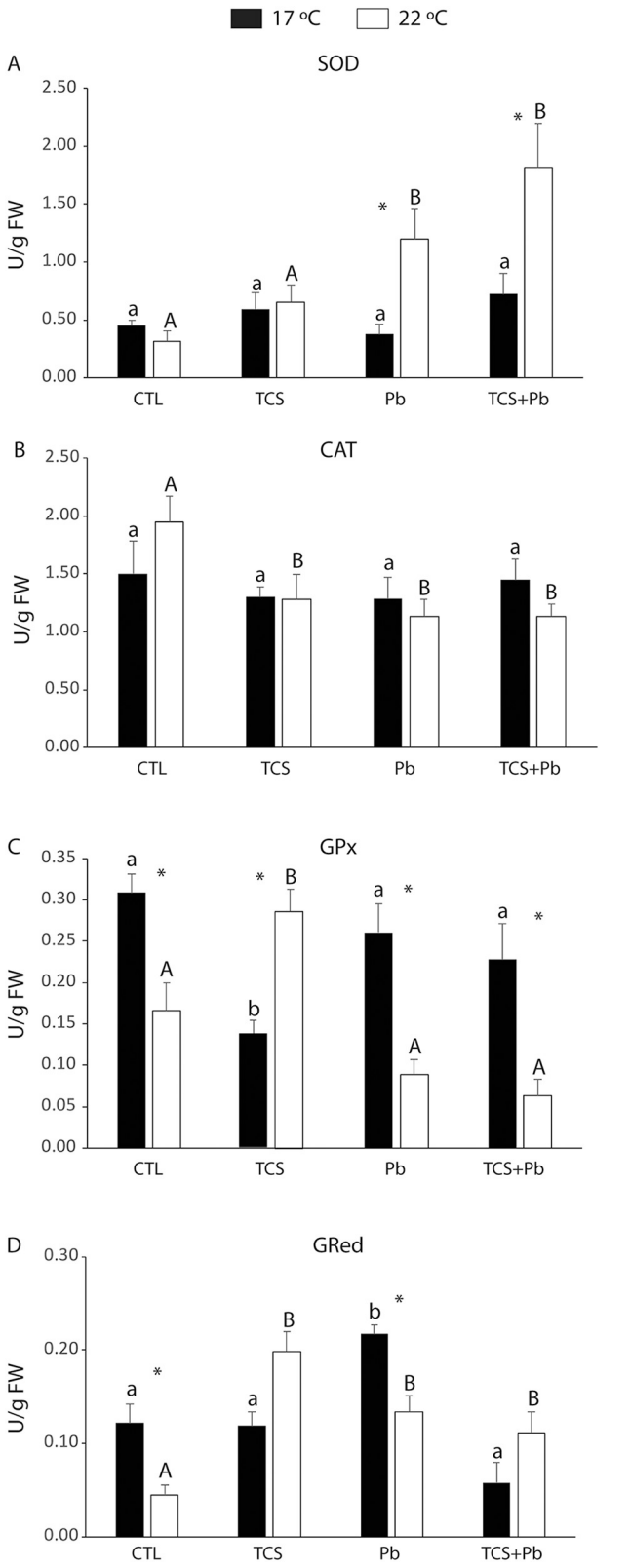

Fig. 2. A) Superoxide dismutase (SOD); B) Catalase (CAT); C) Glutathione peroxidase (GPx) and D) Glutathione reductase (GRed) activities, in *Mytilus galloprovincialis* after 28 days-exposure. At each temperature (17 and 22 °C) the tested treatments and statistical analysis were as in Fig. 1.

3.3. Multivariate analysis

Principal coordinates analysis (PCO) graph for mussels exposed to TCS, Pb and TCS + Pb at two temperatures (17 and 22 °C) is shown in Fig. 5. PCO axis 1 explained 42.3% total variation, while PCO axis 2

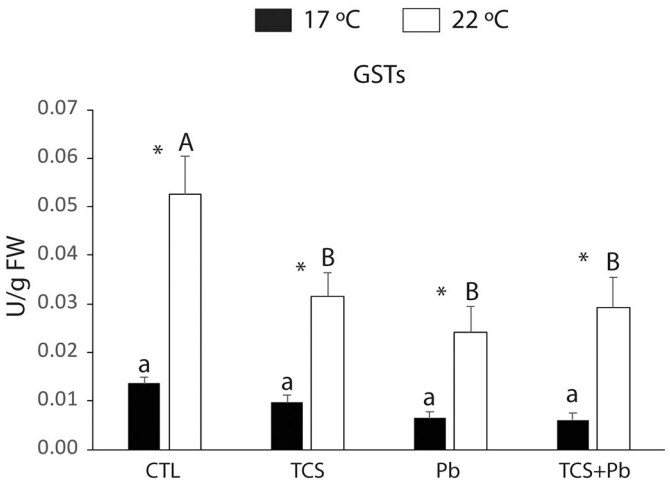

Fig. 3. Glutathione S-transferases (GSTs) activity, in *Mytilus galloprovincialis* after 28 days-exposure. At each temperature (17 and 22 °C) the tested treatments and statistical analysis as in Fig. 1.

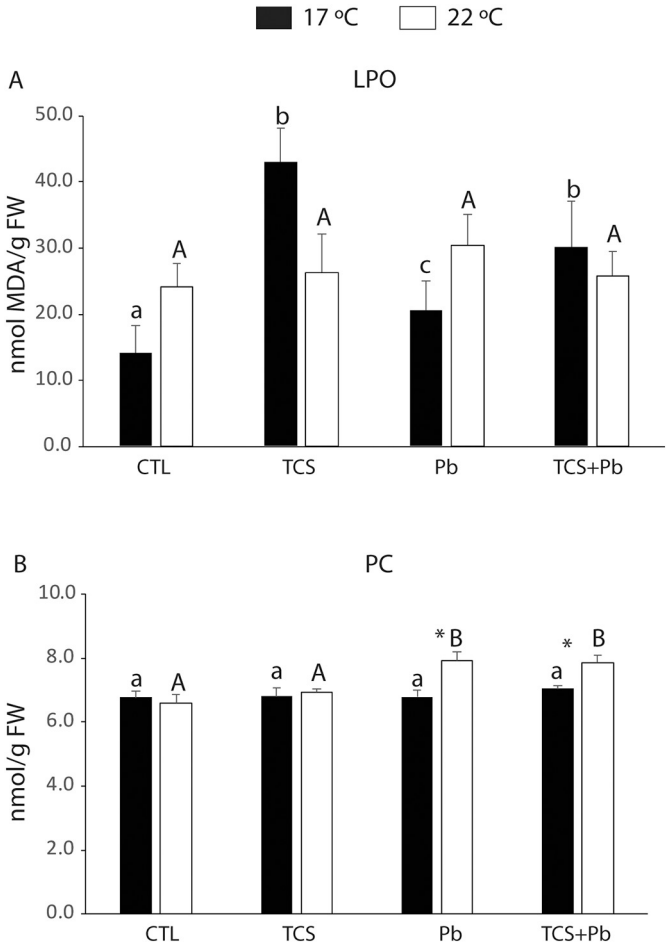

Fig. 4. A) Lipid peroxidation (LPO) and B) protein carboxylation (PC) levels, in *Mytilus galloprovincialis* after 28 days-exposure. At each temperature (17 and 22 °C) the tested treatments and statistical analysis as in Fig. 1.

explained 39.5%. PCO1 separated mussels exposed to temperature 22 °C (CTL, Pb and TCS + Pb) at the positive side from mussels exposed to the remaining treatments in the negative side. Overall PCO2 separates individuals exposed to Pb and 22 °C (Pb and TCS + Pb) at the positive side from the remaining treatments at the negative side. High correlation was observed between GSTs (0.87) and ETS (0.97) and the PCO1 positive side. PC and SOD were highly correlated with PCO2 positive side (0.94 and 0.93, respectively).

4. Discussion

The selected nominal environmentally relevant water concentrations of TCS (1 µg/L) and Pb (50 µg/L) matched the real ones determined by GC–MS (TCS) and AAS (Pb) analysis. Chemical analysis of soft tissue also confirmed the uptake and bioaccumulation of the chemicals in the range of about 10–15 ng/g d.w for TCS and 1–2 µg/g d.w for Pb. The present findings revealed that concentrations of TCS and Pb present in mussels were independent of the testing temperature and, only under the co-exposure conditions Pb was significantly higher at the highest temperature. To relate our measured concentrations in mussels to other studies with bivalves is difficult due to the large variation in levels reported under field situations or even after lab exposures using comparable water concentrations of TCS and Pb. Water concentration of TCS in surface waters can reach up to ≈ 0.5 µg/L (Morrall et al., 2004) and 1 µg/L concentration is frequently selected in bivalves to assess TCS toxicity (Binelli et al., 2011; Kookana et al., 2013; Matozzo et al., 2012). Bioaccumulated concentrations of TCS in *M. galloprovincialis* encountered in the present laboratory exposure are of the same magnitude as those recorded in the same species, located at the outlet of two sewage outlets in Australia (Kookana et al., 2013). As for Pb, the concentrations determined matched those found in bivalves at the sampling area (Ria de Aveiro coast of Portugal) (Freitas et al., 2012) and seen to cause measurable biological effects (Freitas et al., 2014). Comparisons with other studies on aquatic species evaluating the effect of the temperature alone, as a factor controlling bioaccumulation, are highly variable, and even controversial, since they will depend on the species, tissue analysed and experimental conditions. For instance, Coppola et al. (2018) demonstrated that mussels *M. galloprovincialis* exposed to enhanced temperature presented higher As concentration than mussels under control temperature, while when the same species was exposed to Hg an opposite trend was observed with lower Hg concentration in mussels exposed to higher temperature. Nevertheless, Khan et al. (2006, 2007) demonstrated that rising temperature can increase the sensitivity of aquatic animals (namely the crayfish *Orconectes immunis*) and the soil earthworm (*Lumbricus terrestris*) to metals. These authors argued that the increase in toxicity of metals enhanced by high temperature may be due to limiting the scope of aerobic metabolism (oxygen extraction, transport and utilization), since when the metabolism of aquatic species is increased and oxygen concentration in water is reduced, the inflow rate of water into the animal increases in order to extract more oxygen, which can simultaneously increase the entry of dissolved pollutants into the body. Also, Bat et al. (2000) showed that LC_{50} decreased along the increasing temperature in the amphipod *Gammarus pulex pulex* exposed to Cu, Zn and Pb. However, as reviewed by Sokolova and Lannig (2008), metals accumulation in bivalves will highly depend on the element and on the species.

In order to asses if water exposures and consequently bioaccumulated concentrations of TCS and Pb alone and combined at two temperatures had an effect on mussel's physiological performance and toxicity, a comprehensive set of biomarkers were considered. The electron transport system (ETS) activity, commonly selected as a proxy for the metabolic capacity of organisms, revealed that mussels exposed to pollutants decreased their capacity regardless the temperature tested, although the magnitude of the response was greater at 22 °C as control mussels at this temperature already displayed significantly higher basal activity. Lower metabolic activity in pollutant-exposed mussels with respect to controls did not result in a usage of stored energy reserves, especially noticed for GLY, supporting the hypothesis that mussels avoided the expenditure of their energy reserves as a consequence of their reduced metabolism. These results indicate that mussels under stress reduce their metabolism, which could be achieved by closing

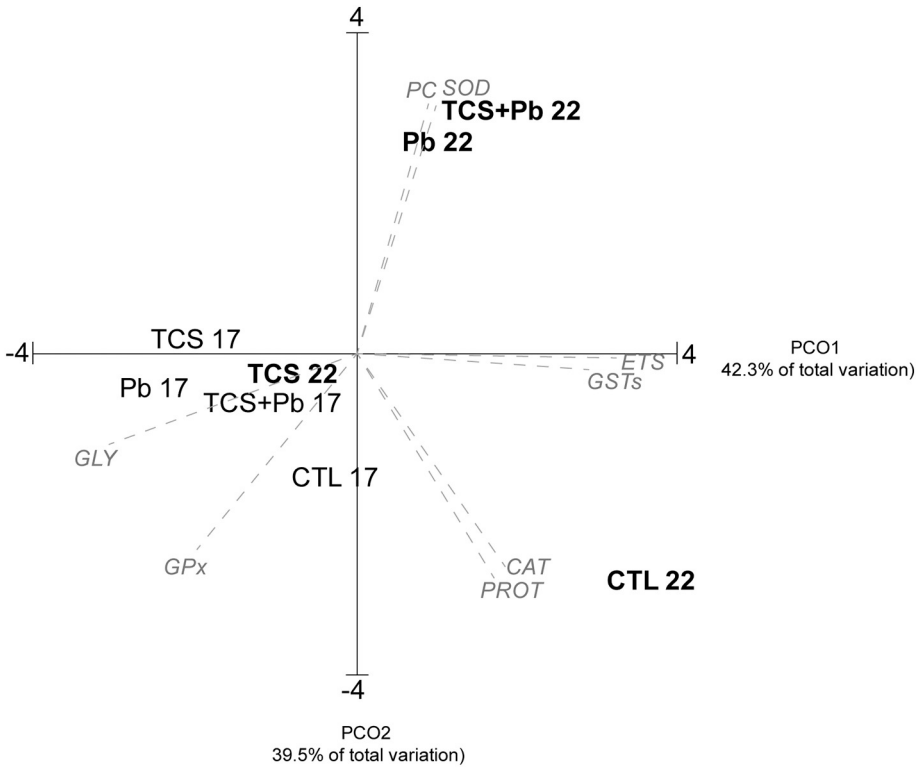

Fig. 5. Centroids ordination diagram (PCO) based on biochemical parameters, measured in *Mytilus galloprovincialis* after 28 days-exposure. At each temperature (17 and 22 °C) the tested treatments were as in Fig. 1. Pearson correlation vectors are superimposed as supplementary variables, namely biochemical data (r > 0.75): PC, SOD, ETS, GSTs, PROT, CAT, GPx, GLY.

their valves, preventing at the same time the bioaccumulation of contaminants, as revealed by similar bioaccumulation of chemicals at both temperatures. It was already demonstrated by other authors that bivalves under stressful conditions use the strategy to close their valves to avoid further damage (Gosling, 2003; Ortmann and Grieshaber, 2003).

Another set of biomarkers to assess chemical exposures concerns to the antioxidant responses and oxidative stress status. The present study revealed that mussels under warming conditions (22 °C) experienced an increase in SOD activity mostly in those exposed to Pb alone and under co-exposure with TCS, while a decrease on CAT activity was revealed under all chemical exposures. At control temperature (17 °C) no responses were observed. Other antioxidant defences such as GPx activity was only affected in mussels exposed to TCS in a temperature dependent way, with an increase in activity at 22 °C and a decrease at 17 °C. Under warming conditions (22 °C) GRed acted in a complementary way to GSTs. That is GRed showed higher activity in exposed mussels while GSTs decreased at this temperature. No response was evidenced at 17 °C in any of these parameters (except for GPx when exposed to TCS and GRed to Pb). Present results indicate that GSTs activity in mussels was more sensible to pollutants under warming conditions.

As a consequence of the overall antioxidant responses, mostly at higher temperature, LPO occurrence was prevented in contaminated mussels at 22 °C. On the other hand, contamination by TCS and Pb and the combination of both stressors induced LPO in mussels at 17 °C coincident with lack of antioxidant responsiveness. Despite the efficiency of enzymatic changes to prevent LPO at 22 °C, PC formation could not be avoided under Pb and co-exposure by TCS, holding Pb as the main responsible for ROS formation and proteins oxidative modifications.

In bivalves, oxidative stress responses and damage have been evaluated after exposure to TCS (Binelli et al., 2011; Matozzo et al., 2012; Riva et al., 2012) and Pb (Franco et al., 2016; Freitas et al., 2014; Shenai-Tirodkar et al., 2017), evidencing that both metabolic capacity and oxidative status may depend on the species and tissues analysed but also on the conditions tested. For example, Matozzo et al. (2012) demonstrated that in the clam *Ruditapes philippinarum* the highest TCS concentrations tested caused a significant increase in SOD activity in gills, but it decreased in digestive gland. Enhanced oxidative stress was induced by Pb in the clam *Ruditapes decussatus* whole tissue (Freitas et al., 2014) as well as in the oyster *Crassostrea madrasensis* (Shenai-Tirodkar et al., 2017). However, we are not aware of any study evaluating the combination of these two factors: chemicals (alone or in mixture) and temperature. Other studies in bivalves have already demonstrated that warming conditions alter the sensitivity of these aquatic species to organic and inorganic pollutants (Attig et al., 2014; Banni et al., 2014; Freitas et al., 2014; Kamel et al., 2012; Moreira et al., 2017; Nardi et al., 2018). Warming alone is also a factor that modulates metabolic and biochemical responses in bivalves, depending on the species, tested temperatures, and length of exposure (Anestis et al., 2007; Coppola et al., 2017; Velez et al., 2017). As an example, Velez et al. (2017) showed that in the clam *R. decussatus* the metabolic capacity, measured as ETS, and the antioxidant defence mechanisms increased at increasing temperature. Other studies such as those of Anestis et al. (2007) showed that the mussel *M. galloprovincialis* increased the duration of valve closure by about six-fold when acclimated to higher temperature (24 °C versus17°C) causing a metabolic depression and probably a shift from aerobic to anaerobic metabolism. Similarly, Coppola et al. (2017) revealed a metabolic depression in *M. galloprovincialis* exposed to increased temperature but still cellular damage was not prevented.

The complexity of the responses that the antimicrobial agent TCS and the toxic metal Pb can exert over the metabolic and oxidative stress of a model bivalve acclimatized to two temperatures during a given period of time (28 days) is particular and difficult to relate to other experimental conditions elsewhere, even from studies also conducted in bivalves (see references above). Too many factors (biological and environmental) can modulate these variables, as indicated before. Thus there is need to find an integrative tool capable of discriminating which environmental factors (chemical and/or temperature) and biochemical parameters are crucial on modulating and responding to particular insults. Thus, we considered the multiparameter PCO tool in the analysis of the responses.

Derived from the chemical analysis and the individual responses for

each parameter to each test compound and the controls included in the PCO integrative model, some conclusions can be outlined. Overall, our study revealed a complex response of mussels exposed to an emerging chemical: TCS and the toxic metal Pb alone and under co-exposure, a situation that will mimic a more realistic environmental case. Moreover, when the temperature factor is put on scene, the complexity of the responses is even larger. The PCO allowed confirmation that temperature was a significant factor to modulate the responses and that these were more evident at 22 °C than at 17 °C with Pb (alone or under co-exposure), seen as the most toxic compound as suggested by PC formation and SOD induction.

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Conflict of interest

The Authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affi liations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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